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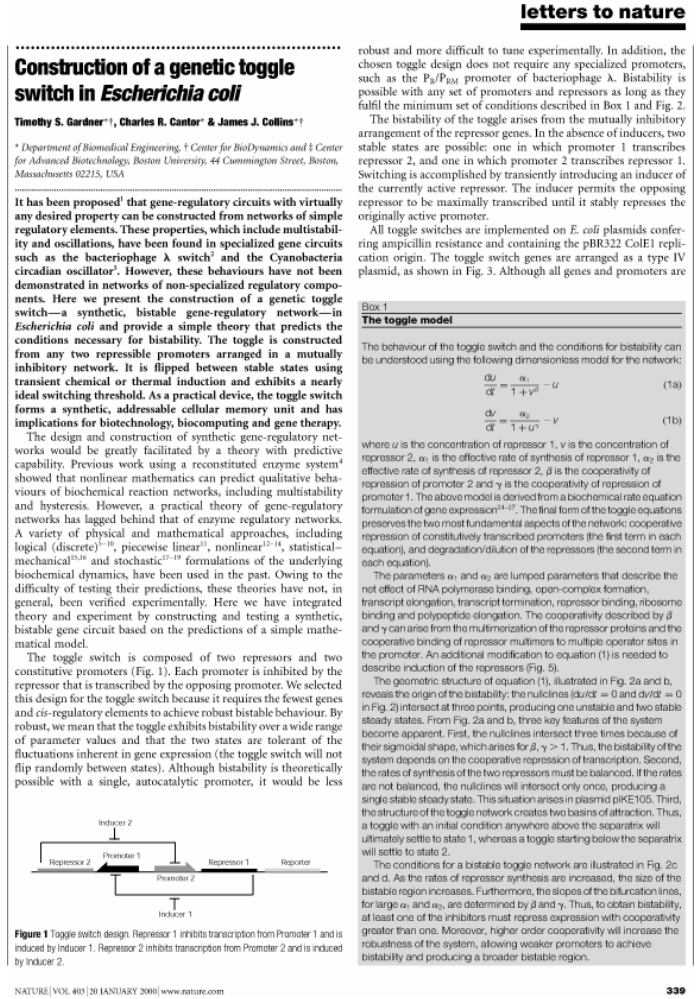

letters to nature

Construction of a genetic toggle switch in *Escherichia coli*

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It has been proposed[1] that gene-regulatory circuits with virtually any desired property can be constructed from networks of simple regulatory elements. These properties, which include multistability and oscillations, have been found in specialized gene circuits such as the bacteriophage λ switch[2] and the Cyanobacteria circadian oscillator[3]. However, these behaviours have not been demonstrated in networks of non-specialized regulatory components. Here we present the construction of a genetic toggle switch—a synthetic, bistable gene-regulatory network—in *Escherichia coli* and provide a simple theory that predicts the conditions necessary for bistability. The toggle is constructed from any two repressible promoters arranged in a mutually inhibitory network. It is flipped between stable states using transient chemical or thermal induction and exhibits a nearly ideal switching threshold. As a practical device, the toggle switch forms a synthetic, addressable cellular memory unit and has implications for biotechnology, biocomputing and gene therapy.

The design and construction of synthetic gene-regulatory networks would be greatly facilitated by a theory with predictive capability. Previous work using a reconstituted enzyme system[4] showed that nonlinear mathematics can predict qualitative behaviours of biochemical reaction networks, including multistability and hysteresis. However, a practical theory of gene-regulatory networks has lagged behind that of enzyme regulatory networks. A variety of physical and mathematical approaches, including logical (discrete)[5–10], piecewise linear[11], nonlinear[12–14], statistical–mechanical[15,16] and stochastic[17–19] formulations of the underlying biochemical dynamics, have been used in the past. Owing to the difficulty of testing their predictions, these theories have not, in general, been verified experimentally. Here we have integrated theory and experiment by constructing and testing a synthetic, bistable gene circuit based on the predictions of a simple mathematical model.

The toggle switch is composed of two repressors and two constitutive promoters (Fig. 1). Each promoter is inhibited by the repressor that is transcribed by the opposing promoter. We selected this design for the toggle switch because it requires the fewest genes and *cis*-regulatory elements to achieve robust bistable behaviour. By robust, we mean that the toggle exhibits bistability over a wide range of parameter values and that the two states are tolerant of the fluctuations inherent in gene expression (the toggle switch will not flip randomly between states). Although bistability is theoretically possible with a single, autocatalytic promoter, it would be less robust and more difficult to tune experimentally. In addition, the chosen toggle design does not require any specialized promoters, such as the P_R/P_{RM} promoter of bacteriophage λ. Bistability is possible with any set of promoters and repressors as long as they fulfil the minimum set of conditions described in Box 1 and Fig. 2.

The bistability of the toggle arises from the mutually inhibitory arrangement of the repressor genes. In the absence of inducers, two stable states are possible: one in which promoter 1 transcribes repressor 2, and one in which promoter 2 transcribes repressor 1. Switching is accomplished by transiently introducing an inducer of the currently active repressor. The inducer permits the opposing repressor to be maximally transcribed until it stably represses the originally active promoter.

All toggle switches are implemented on *E. coli* plasmids conferring ampicillin resistance and containing the pBR322 ColE1 replication origin. The toggle switch genes are arranged as a type IV plasmid, as shown in Fig. 3. Although all genes and promoters are

Figure 1 Toggle switch design. Repressor 1 inhibits transcription from Promoter 1 and is induced by Inducer 1. Repressor 2 inhibits transcription from Promoter 2 and is induced by Inducer 2.

Box 1
The toggle model

The behaviour of the toggle switch and the conditions for bistability can be understood using the following dimensionless model for the network:

$$\frac{du}{dt} = \frac{\alpha_1}{1+v^\beta} - u \quad (1a)$$

$$\frac{dv}{dt} = \frac{\alpha_2}{1+u^\gamma} - v \quad (1b)$$

where u is the concentration of repressor 1, v is the concentration of repressor 2, α_1 is the effective rate of synthesis of repressor 1, α_2 is the effective rate of synthesis of repressor 2, β is the cooperativity of repression of promoter 2 and γ is the cooperativity of repression of promoter 1. The above model is derived from a biochemical rate equation formulation of gene expression[24–27]. The final form of the toggle equations preserves the two most fundamental aspects of the network: cooperative repression of constitutively transcribed promoters (the first term in each equation), and degradation/dilution of the repressors (the second term in each equation).

The parameters α_1 and α_2 are lumped parameters that describe the net effect of RNA polymerase binding, open-complex formation, transcript elongation, transcript termination, repressor binding, ribosome binding and polypeptide elongation. The cooperativity described by β and γ can arise from the multimerization of the repressor proteins and the cooperative binding of repressor multimers to multiple operator sites in the promoter. An additional modification to equation (1) is needed to describe induction of the repressors (Fig. 5).

The geometric structure of equation (1), illustrated in Fig. 2a and b, reveals the origin of the bistability: the nullclines ($du/dt = 0$ and $dv/dt = 0$ in Fig. 2) intersect at three points, producing one unstable and two stable steady states. From Fig. 2a and b, three key features of the system become apparent. First, the nullclines intersect three times because of their sigmoidal shape, which arises for $\beta, \gamma > 1$. Thus, the bistability of the system depends on the cooperative repression of transcription. Second, the rates of synthesis of the two repressors must be balanced. If the rates are not balanced, the nullclines will intersect only once, producing a single stable steady state. This situation arises in plasmid pIKE105. Third, the structure of the toggle network creates two basins of attraction. Thus, a toggle with an initial condition anywhere above the separatrix will ultimately settle to state 1, whereas a toggle starting below the separatrix will settle to state 2.

The conditions for a bistable toggle network are illustrated in Fig. 2c and d. As the rates of repressor synthesis are increased, the size of the bistable region increases. Furthermore, the slopes of the bifurcation lines, for large α_1 and α_2, are determined by β and γ. Thus, to obtain bistability, at least one of the inhibitors must repress expression with cooperativity greater than one. Moreover, higher order cooperativity will increase the robustness of the system, allowing weaker promoters to achieve bistability and producing a broader bistable region.

Design goal and rationale

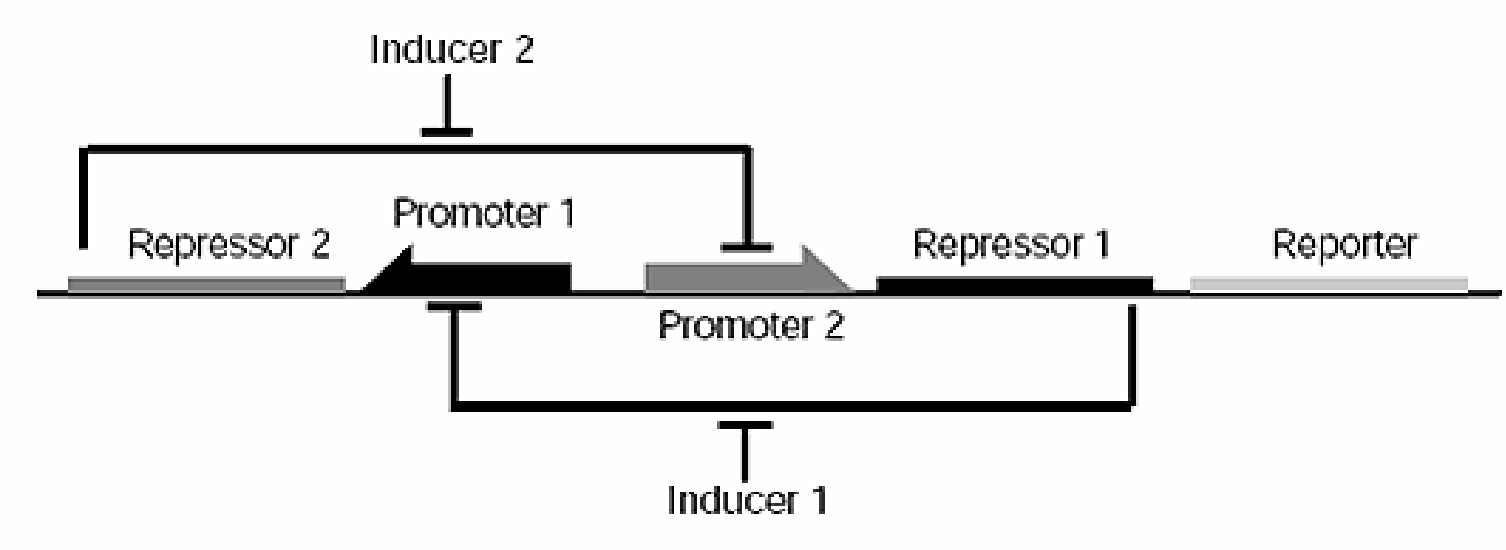
Goal: to integrate theory and experiment by constructing and testing a synthetic, bistable gene circuit based on the predictions of a simple mathematical model.

Build a system requiring the fewest genes and cis-regulatory elements to achieve robust bistable behavior.

Robust means:

- the toggle exhibits bistability over a wide range
- of parameter values
- the two states are tolerant of fluctuations inherent in gene expression (i.e. the toggle switch will not flip randomly between states)

Toggle switch design (Fig. 1)



- Repressor 1 inhibits transcription from Promoter 1 and is induced by Inducer 1.
- Repressor 2 inhibits transcription from Promoter 2 and is induced by Inducer 2.

The toggle switch plasmid (Fig. 3)

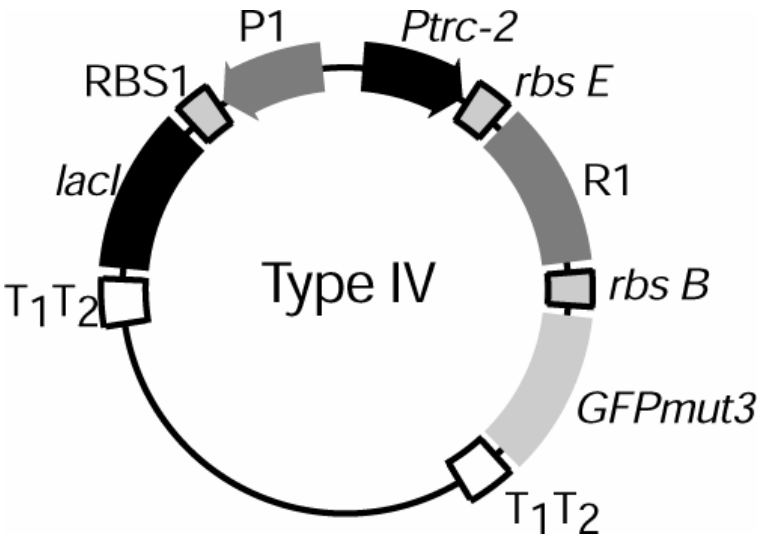


Figure 3 The toggle switch plasmid. Promoters are marked by solid rectangles with arrowheads. Genes are denoted with solid rectangles. Ribosome binding sites and terminators (T1T2) are denoted by outlined boxes. Different P1 promoters, RBS1 ribosome binding sites, and/or R1 repressors, are used for the various toggle switches. Plasmid types I±III, used in the construction and testing of the toggle components, are described in the Supplementary Information.

Modeling equations (Box 1)

$$\frac{du}{dt} = \frac{\alpha_1}{1 + v^{\beta}} - u$$

$$\frac{dv}{dt} = \frac{\alpha_2}{1 + u^{\gamma}} - v$$

u = concentration of repressor 1
v = concentration of repressor 2

α_1, α_2 rate of synthesis of repressor or reporter gene; modified by changing downstream ribosome binding site (RBS)

β cooperativity of repressor of promoter 1
γ cooperativity of repressor of promoter 2

Stability (Fig. 2)

Bistable System Monostable System

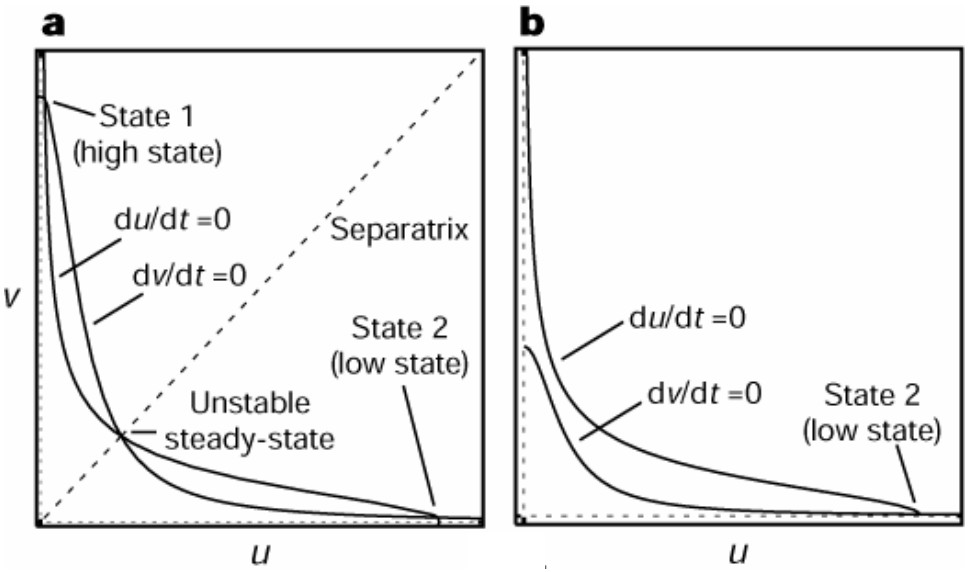
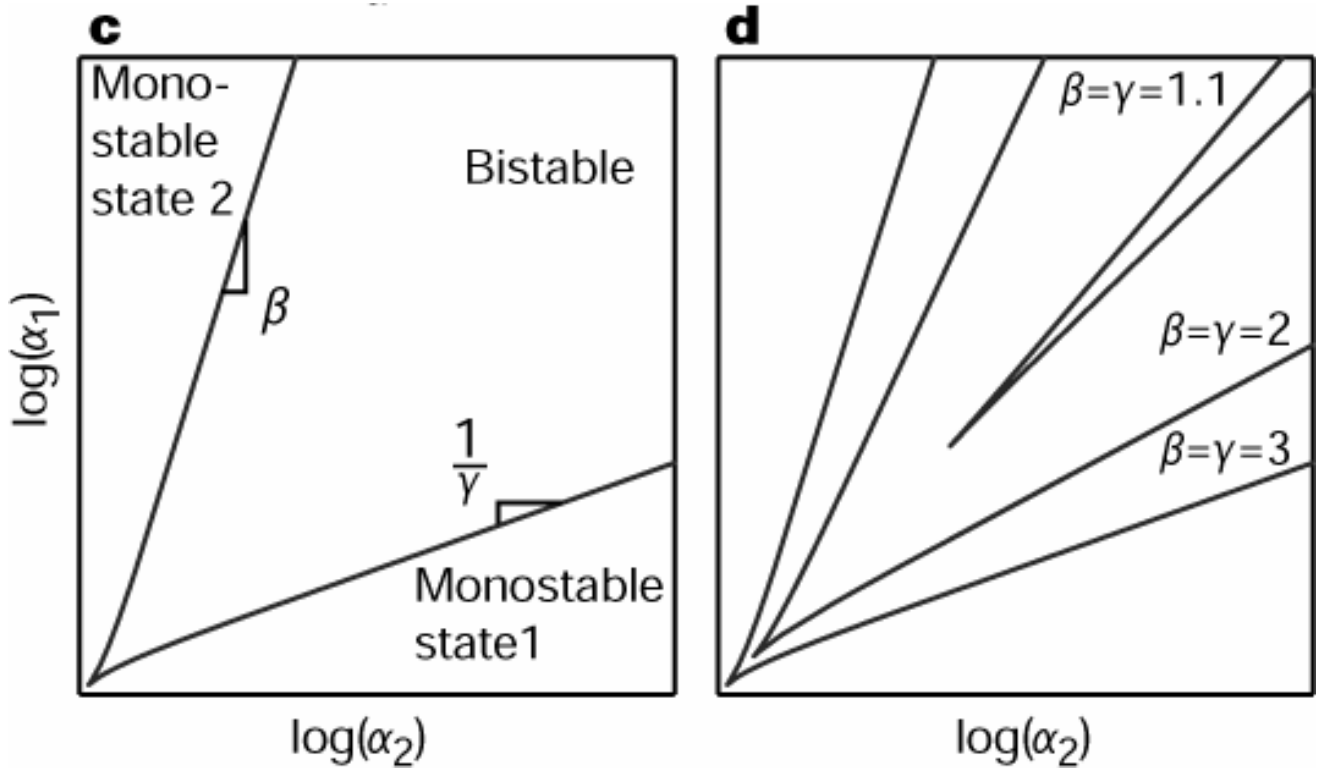


Figure 2 Geometric structure of the toggle equations. **a**, A bistable toggle network with balanced promoter strengths. **b**, A monostable toggle network with imbalanced promoter strengths.

Bistable Parameter Space (Fig. 2)



c, The bistable region. The lines mark the transition (bifurcation) between bistability and monostability. The slopes of the bifurcation lines are determined by the exponents β and γ for large α_1 and α_2. **d**, Reducing the cooperativity of repression (β and γ) reduces the size of the bistable region. Bifurcation lines are illustrated for three different values of β and γ. The bistable region lies inside of each pair of curves.

Plasmids

Class	Promoter 1 (P1)	Repressor 1 (R1)	Promoter 2	Repressor 2
pTAK	P_Ls1con	*cIts*	Ptrc-2	*lacI*
pIKE	$P_L$tetO-1	*tetR*	Ptrc-2	*lacI*

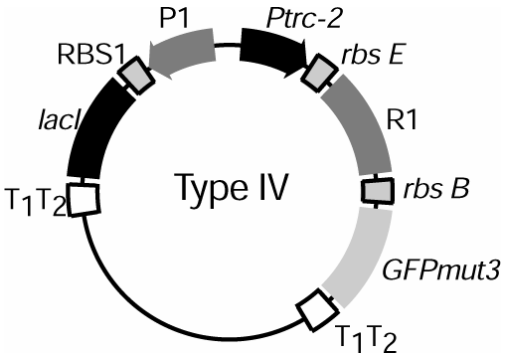
HIGH state = transcription from Ptrc-2 + repression of P1 + GFP

LOW state = transcription from P1 + repression of Ptrc-2

Switch between states

Class	High to Low	Low to High
pTAK	thermal pulse	Isopropyl- β-thiogalactopyranoside (IPTG)
pIKE	Anhydrotetracycline (aTc) pulse	IPTG pulse

Plasmids



Bistable

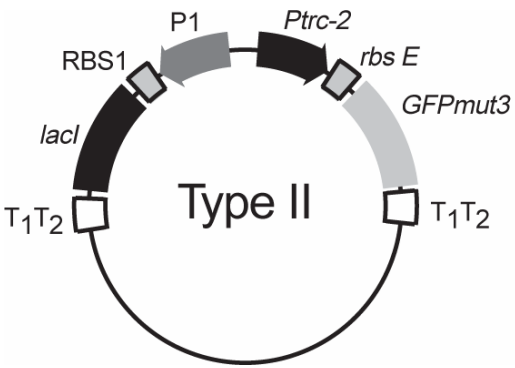
- pTAK117
- pTAK130
- pTAK131
- pTAK132
- pIKE107
- pIKE105

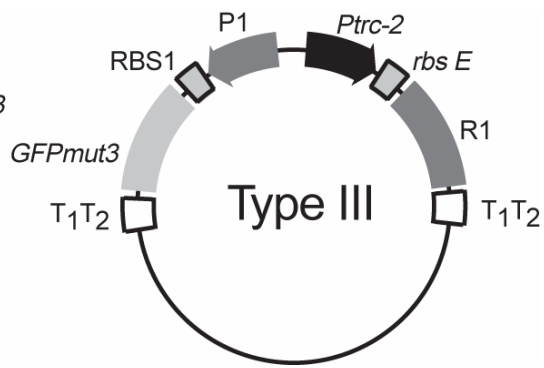
Different RBS1 strength (i.e. different $\alpha 1$, $\alpha 2$)

No bistability

Controls:

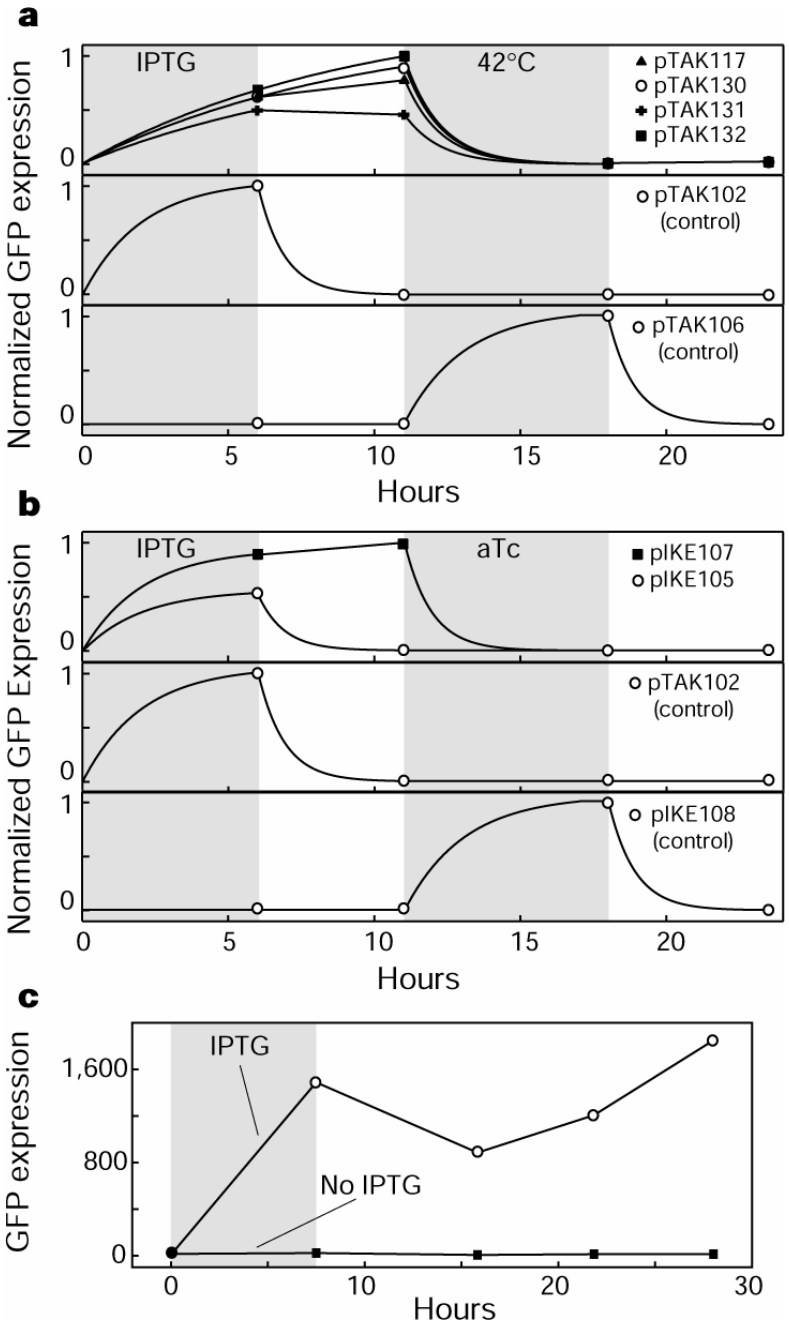
pTAK102





pTAK106
pIKE108

Demonstration of bistability (Fig. 4)



Demonstration of bistability. The grey shading indicates periods of chemical or thermal induction. The lines in a and b, which are approximations of the switching dynamics, are included for clarity. **a**, pTAK toggle plasmids and controls. **b**, pIKE toggle plasmids and controls. **c**, demonstration of the long-term stability of the separate expression states in pTAK117.

Toggle switch induction threshold (Fig. 5a)

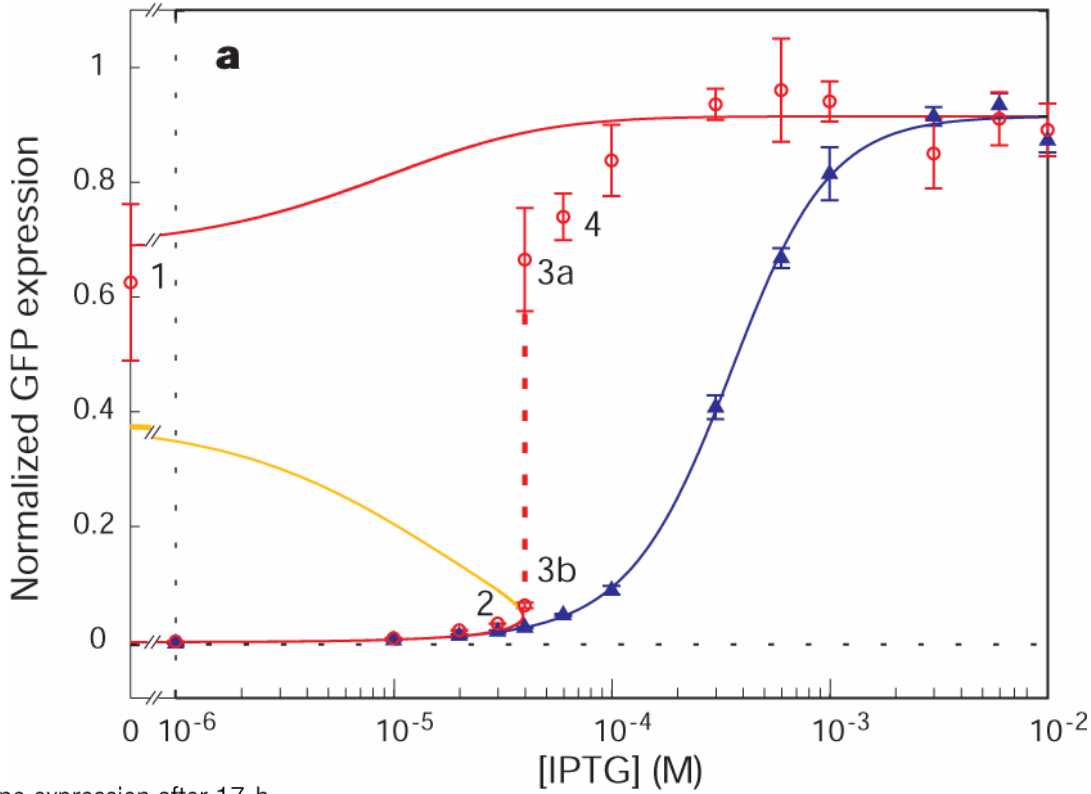
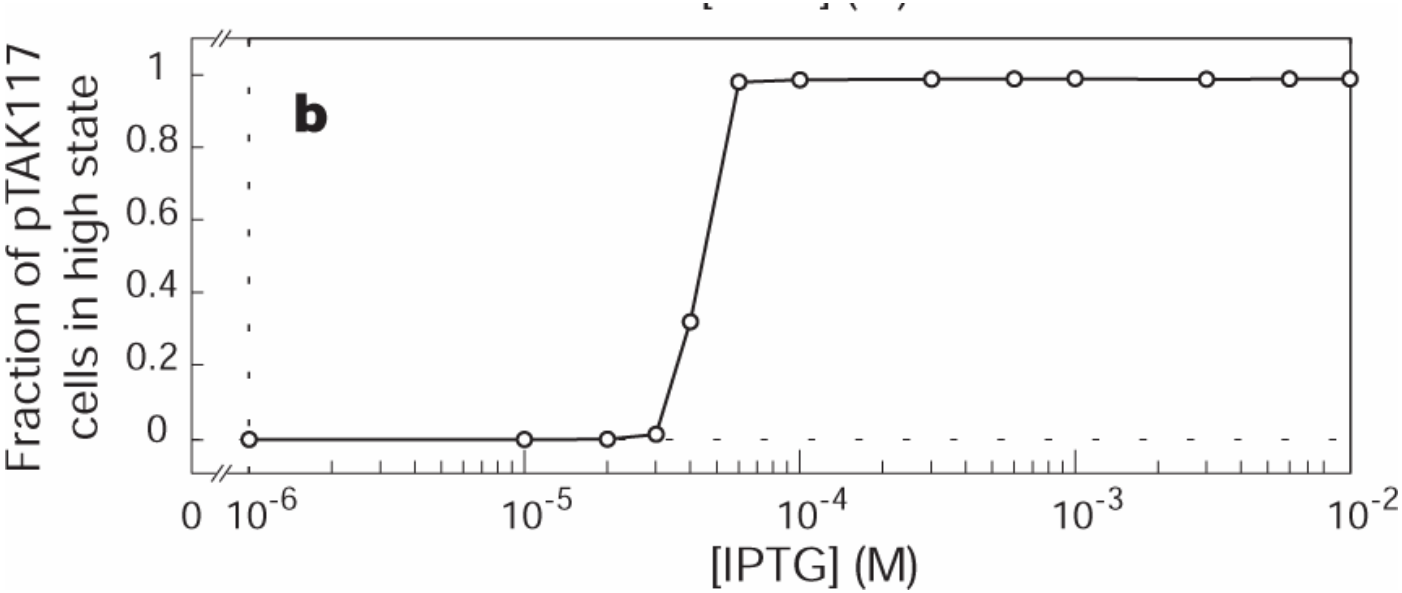


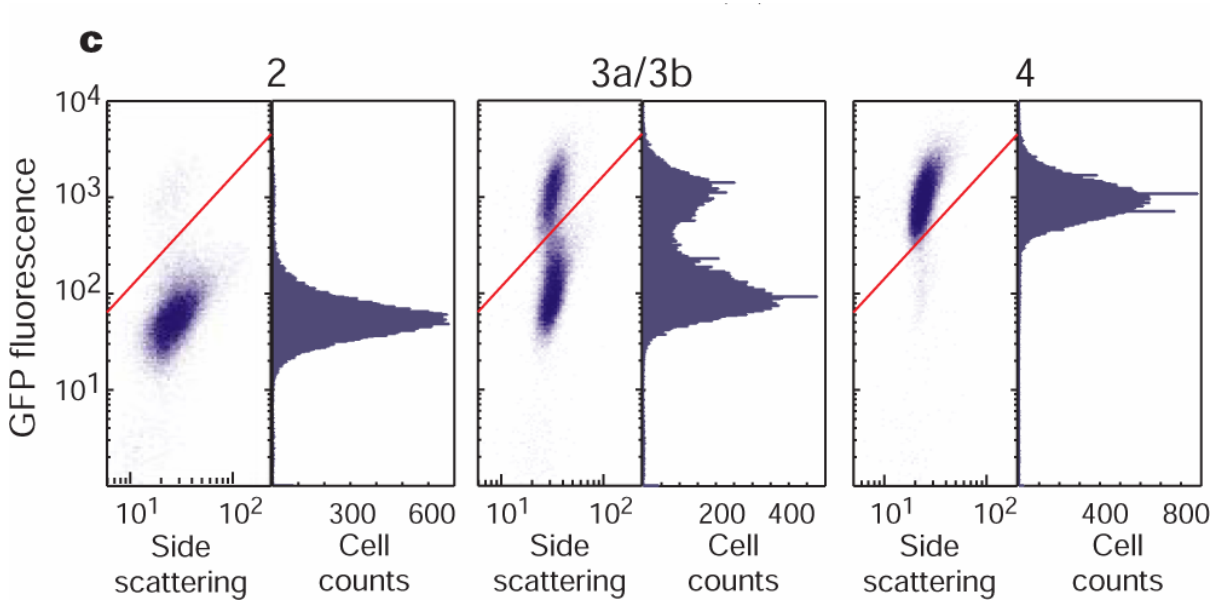
Figure 5 Toggle switch induction threshold. **a**, Steady-state gene expression after 17-h induction. The pTAK117 toggle plasmid (red circles) exhibits a quasi-discontinuous jump to the high state whereas the pTAK102 control plasmid (blue triangles) exhibits a sigmoidal induction curve. Point 1 is taken from separate experiments measuring the high state of pTAK117 with no inducer. Points 3a and 3b are the high and low modes of a bimodally distributed cell population. The bimodality occurs due to natural fluctuations in gene expression and the close proximity of the toggle switch to its bifurcation point. Theoretical curves are calculated from equation (1) with the term $u/(1 + [\text{IPTG}]/K)^{\eta}$, where K is the dissociation constant of IPTG from LacR and η is the cooperativity of IPTG binding, replacing u in the denominator of equation (1b). The red curves show the stable steady states and the orange curve shows the unstable steady state of the toggle. The blue curve shows the steady state of the IPTG-inducible control plasmid. Model parameters for the theoretical curves are $\alpha_1 = 156.25$, $\alpha_2 = 15.6$, $\beta = 2.5$, $\gamma = 1$, $\eta = 2.0015$, $K = 2.9618 \times 10^{-5}$. **b**, Fraction of toggle cells in the high state at various concen-

Toggle switch induction threshold (Fig. 5b)



$K = 2.9618 \times 10^{-5}$. **b**, Fraction of toggle cells in the high state at various concentrations of IPTG. The sudden switching to the high state is more clearly visible. High and low cell populations were divided as described for **c** below. **c**, Scatter plots (left plots) and

Toggle switch induction threshold (Fig. 5c)



low cell populations were divided as described for **c** below. **c**, Scatter plots (left plots) and histograms (right plots) illustrating the condition of the toggle cells at points 2, 3 and 4 (of **a**) near the bifurcation point. High-state and low-state cell populations are divided by the red line in the scatter plots. The two states of the toggle are clearly evident in the bimodally distributed cells (point 3).

pTAK117 switching time (Fig.6)

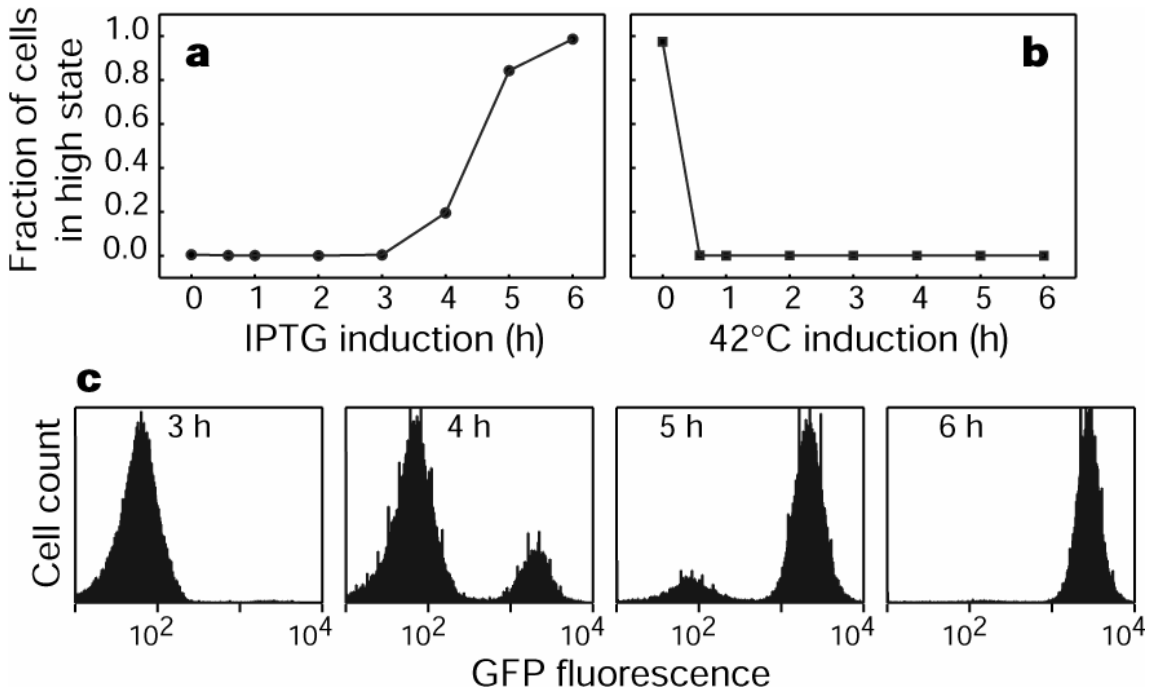


Figure 6 pTAK117 switching time. **a**, **b**, The fraction of cells in the high state is plotted as a function of the induction time. Cells were divided between high and low states as in Fig. 5c. **c**, Switching of pTAK117 cells from the low to the high state by IPTG induction. The cell population is illustrated at four time points. Cells begin switching between 3 and 4 h as shown by the appearance of a bimodal distribution. The switching is complete by 6 h.

Fig. 1, supplement

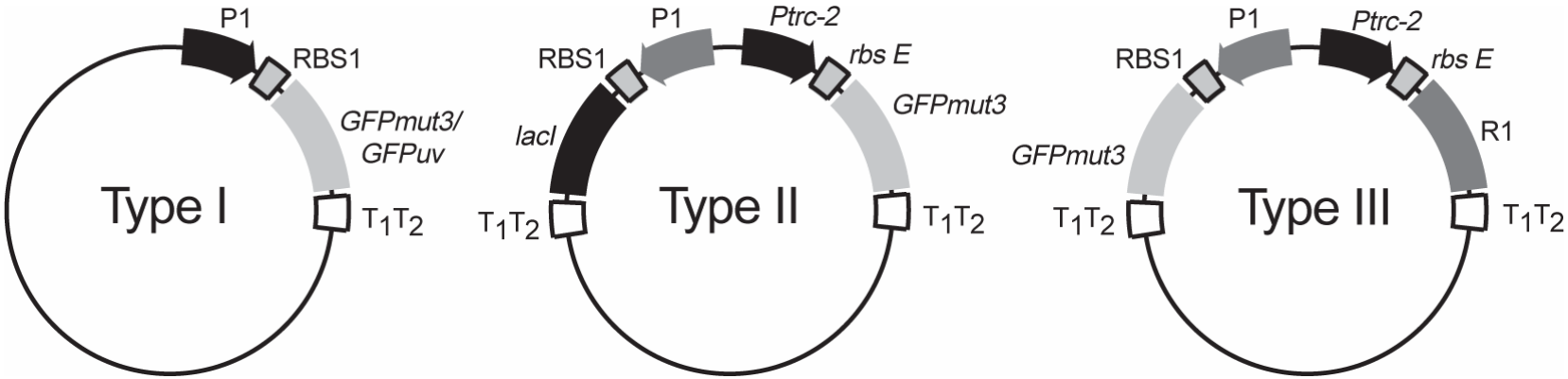


Figure 1: Promoters are marked by solid rectangles with arrowheads. Genes are denoted with solid rectangles. Ribosome binding sites and terminators ($T_1\,T_2$) are denoted by outlined boxes. The Ptrc-2 promoter with RBS-E and the lacI gene is used in all Type II and III plasmids.

Table 1, supplement

Plasmid	Type	P1	RBS1	RBS2	GFP Expression
Bare Promoters					
pMKN7a*	I	Ptrc-2	E	—	732 ± 108
pBAG102	I	$P_L$tetO-1	C	—	5.5 ± 0.1
pBAG103	I	$P_L$tetO-1	A	—	660 ± 42
pBRT21.1*	I	P_Ls1con	D	—	9,390 ± 840
pBRT21.1*†	I	P_Ls1con	D	—	14,300 ± 400
pBRT123	I	P_Ls1con	G	—	387 ± 21
pBRT124	I	P_Ls1con	F	—	972 ± 43
pBRT125	I	P_Ls1con	H	—	15.9 ± 3.2
lacI Repression					
pTAK102	II	P_Ls1con	D	—	36.0 ± 3.8
pTAK103a	II	P_Ls1con	G	—	137 ± 8
cI Repression					
pTAK106	III	P_Ls1con	D	—	2.5 ± 0.3
pTAK107	III	P_Ls1con	G	—	2.0 ± 0.1
tetR Repression					
pIKE108	III	$P_L$tetO-1	A	—	5.8 ± 1.0
pIKE110	III	$P_L$tetO-1	C	—	2.3 ± 0.2
Toggles					
pTAK117	IV	P_Ls1con	D	B	bistable
pTAK130	IV	P_Ls1con	G	B	bistable
pTAK131	IV	P_Ls1con	F	B	bistable
pTAK132	IV	P_Ls1con	H	B	bistable
pIKE105	IV	$P_L$tetO-1	A	B	monostable
pIKE107	IV	$P_L$tetO-1	C	B	bistable

*Estimated from flow-cytometer assay of GFPuv-expressing promoters.
†Grown at 32°C.

Fig. 2, supplement

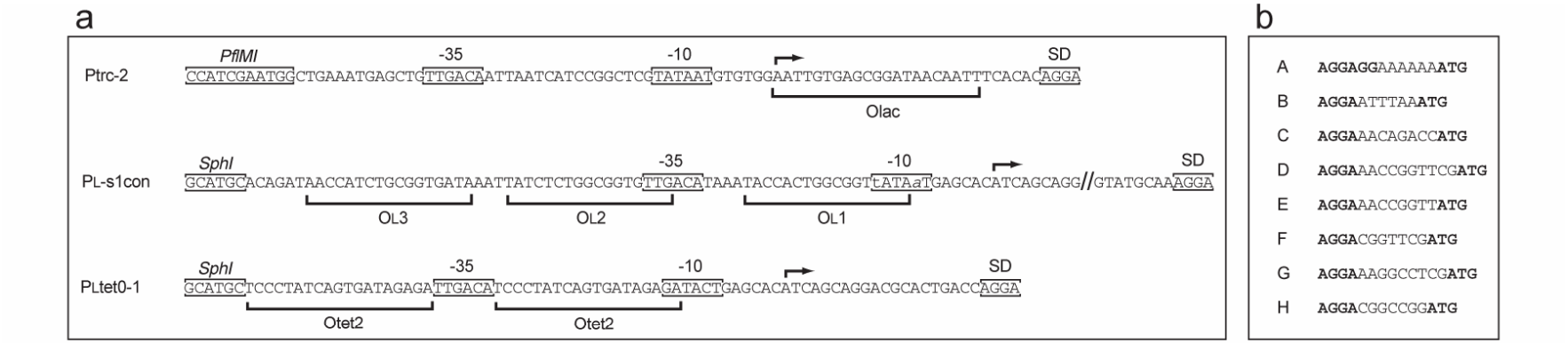


Figure 2: Promoters and ribosome binding sites used to construct the toggle plasmids. **a**, *Promoters. The upstream limit of each promoter is marked by the indicated restriction site. Operator sites are marked be a single underbracket. The initiation of transcription is denoted with arrows. SD denotes the Shine-Dalgarno sequence. Mutations in the -10 sequence of* P_L *s1con are indicated with lowercase letters.* **b**, *Ribosome binding sites. Shine-Dalgarno sequences and start codons are in boldface. Sequences are ranked in order of their translational efficiency (A = highest, G = lowest).*